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Award Number: W81XWH-06-1-0649

TITLE: Improving Breast Cancer Diagnosis by Antisense Targeting

PRINCIPAL INVESTIGATOR: Yi Wang, Ph.D.

CONTRACTING ORGANIZATION: University of Massachusetts Medical School
Worcester, MA 01655

REPORT DATE: Aug 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
1-08-2007	Annual	15 JUL 2006 - 14 JUL 2007

4. TITLE AND SUBTITLE

Improving Breast Cancer Diagnosis by Anitsense Targeting

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0649

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Yi Wang, Ph.D,

E-Mail: Yi.Wang@umassmed.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Massachusetts Medical School
Worcester, MA 01655

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of this investigation is to using anti-Her2 antibody Herceptin as carrier and streptavidin as linker to specifically transport antisense into tumor cells expressing Her2. For this purpose, we successfully conjugated MAG3/biotin to antisense/sense morpholino oligomers (MORFs), and conjugated biotin group to Herceptin. The MORF-streptavidin-Herceptin constructs have been synthesized and their quality has been confirmed. We have confirmed by confocal microscopy using fluorescent lissamine as the tag that Herceptin can mediate the cellular internalization of MORFs, and more important, the internalized oligomer distributed in cytoplasm evenly without apparent entrapment in cellular compartments. By using 99mTc as the tag, we also have approved that the antisense can be released from the internalized antisense-streptavidin-Herceptin construct. These results are significant and encouraging for the followed studies in in vivo antisense tumor targeting using Herceptin as carrier.

15. SUBJECT TERMS
Antisense tumor targeting, Her2 antigen, Herceptin, antibody tumor targeting

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	17	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction

This study is focused on early detection of breast cancers with propensity to metastasize. This work can also be considered for antisense radiotherapy if successful by seamless substitution of the imaging radionuclide with a therapeutic radionuclide. When administrated intravenously, the high affinity of anti-HER-2 antibody to HER-2 expressed on surface of breast tumor cells will image not only the primary tumors, but also metastases throughout the patient including nodal metastases usually bypassed by conventional methods. The combination of anti-HER-2 tumor targeting, radiolabeled anti-RhoC mRNA antisense oligomers and nuclear imaging will improve early diagnosis, detection of metastases, and outcome prediction in patients with aggressive breast cancers. The advantages of the method proposed in this study are: (1) it targets HER-2 expressing primary and metastatic breast cancers including nodal metastases; (2) the background can be effectively reduced by injection of D-biotin; (3) if successful this technology may be used for both tumor early diagnosis and outcome prediction to avoid invasive surgical methods. We will apply our extensive experience in antisense targeting of cancers to improve the detection of breast cancers by this novel approach. If successfully developed, this non-invasive imaging technology will be superior to the conventional approaches requiring invasive surgery. We expect the method can be used eventually to produce a variety of antisense-carrier constructs targeting different oncogene mRNAs.

Body

In our proposal we outlined the following studies to be performed: (1) To construct the antibody-antisense oligomer conjugate using streptavidin as linker. (2) To demonstrate the overexpression of RhoC mRNA in SUM-149 and 190 cells in culture and targeting of the antisense MORF to this mRNA. (3) To confirm internalization of the antibody when conjugated to streptavidin-MORF. (4) To detect by noninvasive imaging by an antisense mechanism in mouse tumor models primary tumors and metastases.

The following describes our achievements in the past year (July 2006~July 2007), the unpublished data are underlined.

(1) We successfully conjugated NHS-MAG3 group to antisense and sense MORFs, which were commercially obtained with a biotin group on the 3' end, through the primary amine group on the 5' end. The radiolabeling efficiency with ^{99m}Tc was more than 90% confirmed by size exclusion HPLC, a quality sufficient for our in vitro and in vivo studies. In our preliminary studies, we used biotinylated peptides tat and polyarginine as model molecules and successfully developed the method of using streptavidin as linker to form MORF/streptavidin/carrier conjugates at a 1:1:1 molar ratio. The fact that biotinylated tat and polyarginine were chosen as model molecules before going to biotinylated Herceptin was to simplify the synthesis since both the biotinylated cell penetrating peptides were commercially available with high quality and were in stock in our lab. Part of these work resulted a paper entitled "Simplified preparation via streptavidin of antisense oligomers/carriers nanoparticles showing improved cellular delivery in culture", which has been published in Bioconjugate Chemistry (*Bioconjug Chem* 18:1338-43, 2007) and two conference abstracts (please refer to REPORTABLE OUTCOMES**)**.

Afterwards, we synthesized the biotinylated Herceptin and the reaction conditions were strictly controlled and resulted in a product with group per molecule (GPM) of 1.03, i.e. each Herceptin molecule has 1.03 biotin groups on average.

The conjugation of biotin group to Herceptin requires comment. The GPM should be strictly controlled to avoid any crosslinking during the preparation of MORFs/streptavidin/Herceptin. In addition, because we found that the length and the nature of the linker between biotin and Herceptin influences greatly the kinetics of the binding of biotinylated Herceptin to streptavidin, to obtain the best results, a linker of at least 20 angstroms in length is required.

Based on the method by using biotinylated tat and polyarginine as model molecules, we successfully synthesized the ^{99m}Tc labeled MORFs/streptavidin/Herceptin conjugates at a molar ratio of 1:1:1 and the quality of these conjugates were confirmed by size exclusion HPLC.

(2) By RT-PCR, we confirmed that the overexpressions of RhoC mRNA in SUM-149 and 190 cells in culture are at a similar level, as shown in Figure 1. The procedure of RT-PCR was as following: Total RNA was extracted using the RNeasy® Mini extraction kit (Qiagen, Valencia, CA) and the RT-PCR was performed using QIAGEN one-step RT-PCR kit. The final reaction mixture contained 2.5 mM Mg^{2+}, 200 µM dNTP and 0.4 µM of each primer. Reverse-transcription was performed at 50°C for 30 min. Reaction mixtures were heated to 95°C for 15 min to activate HotStarTaq, then PCR amplification was performed for 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 60 s, followed by final extention at 72°C for 10 min. The amplification products were electrophoresed on a 1.0% agarose gel containing 0.02% ethidium bromide and photographed. The β-actin mRNA was used as reference.

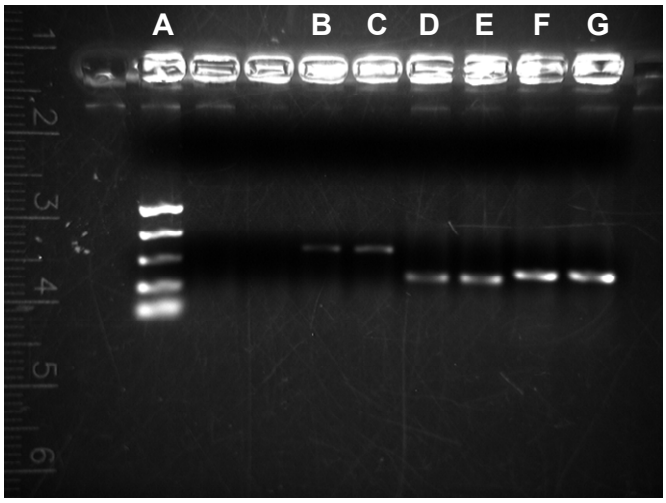

Figure 1 Agarose gel electrophoreses of RT-PCT products of RhoC, RIα and β-actin mRNAs in SUM149 and SUM190 cells. Lane A: molecular marker; Lane B and C: RhoC mRNA; Lane D and E: RIα mRNA; Lane F and G: β-actin mRNA. Lane B, D, and F: SUM149 cells; Lane C, E and G: SUM190 cells. The β-actin mRNA was used as internal reference.

(3) To confirm internalization of the antibody when conjugated to streptavidin-MORF, we commercially obtained antisense and sense MORFs with a fluorophore lissamine group on their 3' end and a primary amine group on their 5' end. Through the primary amine group we successfully conjugated a biotin group to the 5' end of both antisense and sense MORFs thus providing us with the required biotin and fluorophore conjugated oligomers needed for the constructions of fluorescence MORFs/streptavidin/Herceptin. The GPM was optimized to 1.01. The fluorescence MORFs/streptavidin/Herceptin were then synthesized.

Both SUM190 and SUM149 cells were incubated with fluorescence MORFs/streptavidin/Herceptin for 6 h, and the live cells were viewed under confocal microscope. As an example, the following images (Figure 2) present a comparison by live cell confocal microscopy of cellular accumulations of lissamine conjugated antisense MORF/streptavidin/Herceptin in SUM190 (study) and SUM149 (control) cells. The red color indicates the subcellular distribution of antisense MORF oligomer and the blue color indicates the nuclei stained with Hoechst 33342 . As shown, the HER-2 negative SUM149 control cells have almost no accumulation of antisense oligomer. The results show that the Herceptin can efficiently mediate cellular delivery of antisense MORF oligomer into SUM190 cells without entrapment in vesicles.

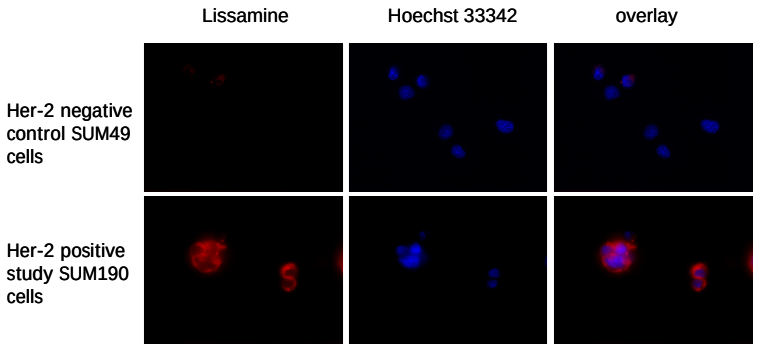

Figure 2 Herceptin improves the delivery of antisense MORF into HER-2 positive SUM190 cells but not into HER-2 negative control SUM149 cells. Cells were incubated with fluorescence antisense MORF/streptavidin/Herceptin for 6 h. Thereafter media were removed the cells were washed with PBS and further incubated with Hoechst 33342 for nuclei staining. Then cells were viewed under confocal microscope.

By using the ^{99m}Tc labeled MORFs/streptavidin/Herceptin conjugates instead of the fluorophore labeled MORFs/streptavidin/Herceptin conjugates, we studied the fate of the MORF once internalized into cells by analyzing cell lysates by size exclusion HPLC. This analysis is capable of determining whether the MORFs/streptavidin/Herceptin conjugates remains intact or whether the MORF is released to target its mRNA. Our conclusion is that the internalized antisense oligomer mediated by Herceptin is released slowly from the MORF/streptavidin/Herceptin complex, presumably an essential step necessary to permit the antisense MORF access to its target mRNA. These studies resulted in a conference abstract entitled "The accumulation of antisense MORF is improved when linked to internalizing antibodies via streptavidin and with evidence of release", which has been accepted as a poster to the *Society for Molecular Imaging and Academy of Molecular Imaging Joint Molecular Imaging Conference* (September

2007, Providence, Rhode Island, USA). The evidence of release of the antisense MORF in cells is shown in Figure 3.

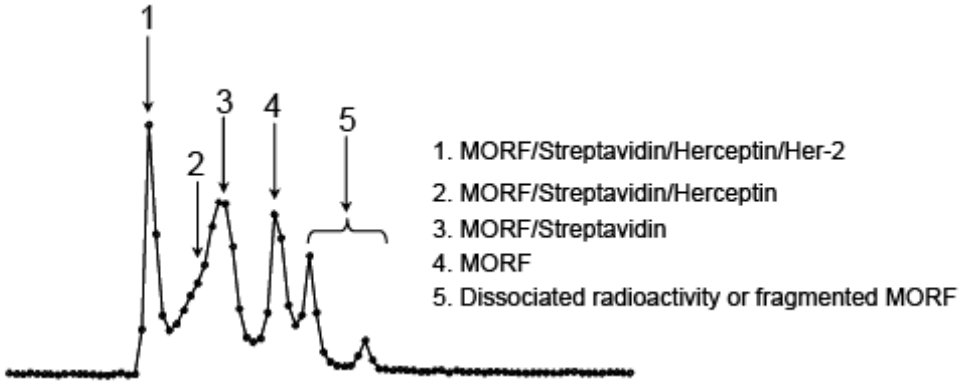

Figure 3 The evidence that the internalized antisense MORF was released. SUM190 cells were incubated with MORF/streptavidin/Herceptin at 10 nM for 6 h. Then the cells were washed first with PBS twice then with 0.2 M glycine-HCl buffer (pH 2.3, containing 1% DMSO) for 2 min to remove cell membrane bound MORF/streptavidin/Herceptin. The cells were lyzed by lysis buffer (10 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.6). After centrifuged at 12000 rpm for 10 min, aliquot of the supernatant (clear lysate) was analyzed on size HPLC.

Key Research Accomplishments

(1). We successfully constructed the antibody-antisense oligomer conjugate using streptavidin as linker.
(2). We demonstrated the overexpression of RhoC mRNA in SUM-149 and 190 cells in culture.
(3). We confirmed increased accumulation into HER-2 positive SUM190 cells but not into HER-2 negative control of the antisense MORF when attached to Hereceptin by streptavidin
(4). We confirmed internalization of the antibody when conjugated to streptavidin-MORF, a necessary condition for antisense targeting.

Reportable Outcomes

Paper published:
Yi Wang, Kayoko Nakamura, Xinrong Liu, Kitamura N, Atsushi Kubo, Donald J Hnatowich. Simplified preparation via streptavidin of antisense oligomers/carriers nanoparticles showing improved cellular delivery in culture. *Bioconjug Chem* 18:1338-43, 2007.

Paper in preparation:
Yi Wang, Xinrong Liu, Kayoko Nakamura, Ling Chen, Mary Rusckowski and Donald J Hnatowich. Evidence for intracellular instability of a tat peptide.

Conference abstracts:

(1). Yi Wang, Xinrong Liu, Kayoko Nakamura, Mary Rusckowski and Donald J Hnatowich. Evidence for intracellular instability of a tat peptide. Poster presentation on 54th SNM Annual Meeting. June 2007, Washington DC. J Nucl Med Suppl. 48: 322p.

(2). Yi Wang, Kayoko Nakamura, Xinrong Liu, Atsushi Kubo and Donald J Hnatowich. Streptavidin linked antisense MORF/carrier nanoparticles showing improved cellular delivery in culture. Oral presentation on 54th SNM Annual Meeting. June 2007, Washington DC. J Nucl Med Suppl. 48: 181p.

(3). Yi Wang, Xinrong Liu, Kayoko Nakamura, Atsushi Kubo, Donald J Hnatowich. The accumulation of antisense MORF is improved when linked to internalizing antibodies via streptavidin and with evidence of release. Accepted as poster by *Joint Molecular Imaging Conference*. September 2007, Providence, Rhode Island, USA.

Conclusion

Using Herceptin (more general, internalizing antibodies) as carrier, the MORF oligomers with sequence sense and antisense to target mRNA can be delivered efficiently into tumor cells expressing HER-2 (more general, antigens), and the antisense oligomer can be slowly released in cytoplasm without entrapment in cellular vesicles, which is an essential step for antisense MORF to bind to its target mRNA. However, although the results obtained so far are very encouraging, whether the released antisense MORF can bind to its target mRNA and whether the strategy can be used for in vivo antisense tumor targeting needs further investigation.

References

None

Appendices……

Appendix I. Abstract presented as poster on 2007 SNM conference in Washington DC.

Appendix II. Abstract presented as oral presentation on 2007 SNM conference in Washington DC.

Appendix III. Abstract accepted as poster to the Society for Molecular Imaging and Academy of Molecular Imaging Joint Molecular Imaging Conference in Rhode Island.

Appendix IV. Paper published in Bioconjugate Chemistry.

Appendix I. Abstract presented as poster on 2007 SNM conference in Washington DC. J Nucl Med Suppl. 48: 322p.

Evidence for intracellular instability of a tat peptide

Y. Wang[1], X. Liu[1], K. Nakamura[2], M. Rusckowski[1], D. J. Hnatowich[1]

1. Radiology, University, of Massachusetts Medical School, Worcester, Massachusetts; 2. Radiology, Keio University School of Medicine, Tokyo, Japan.

Objectives: To investigate the intracellular fate of a tat peptide and provide the first evidence of stability in connection with the delivery of antisense DNAs and other cargos requiring cellular transport. **Methods**: The biotinylated tat peptide (GRKKRRQRRR) was conjugated with MAG3 and radio labeled with Tc99m. The labeled tat was incubated at 20 nM with MKN45 cells at 37°C and the cellular accumulations measured over time. The identical incubation in cell free media was performed simultaneously. Aliquots of both media were removed at 3 to 12 h and analyzed by size exclusion HPLC before and after addition of streptavidin. The absence of low molecular weight peaks and a shift in the radioactivity profile to shorter elution times after the addition of streptavidin both indicate Tc99m-tat stability. **Results**: After an initial rapid accumulation, the cellular radioactivity decreased by about 90% between 1 and 12 h. While the HPLC analysis showed no change in the Tc99m-tat peptide incubated in cell-free media, in the presence of cells, only 50% of the Tc99m was on intact tat after 12 h and less over time. The analysis also showed that the remaining radioactivity was on shorter peptide fragments without a biotin moiety.
Conclusions: The Tc99m-tat was stable in media under the conditions of this investigation and accumulated in MKN45 cells rapidly. However, the Tc99m was also rapidly released from cells as radiolabeled peptide fragments probably generated by intracellular degradation. That the steady-state cell accumulation was never more than 3% of the added Tc99m-tat yet more than 50% of the Tc99m-tat was degraded after 12 h requires that the influx, degradation and efflux of the radiolabeled fragments were continuous. This intracellular instability may explain the efficacy of this cell penetrating peptide as a transmembrane carrier if its instability permits intracellular release of its cargo.

Appendix II. Abstract presented as oral presentation on 2007 SNM annual conference in Washington DC. J Nucl Med Suppl. 48: 181p.

Streptavidin linked antisense MORF/carrier nanoparticles showing improved cellular delivery in culture

Y. Wang[*1], K. Nakamura[2], X. Liu[1], A. Kubo[2], D. J. Hnatowich[1]

1. Radiology, University of Massachusetts Medical School, Worcester, Massachusetts; 2. Radiology, Keio University School of Medicine, Tokyo, Japan.

Objectives: Recognizing that streptavidin may be a convenient linker between biotinytated carriers for improved delivery and antisense oligomers that would require only simple mixing for preparation, we have prepared nanoparticles combining carriers with an antisense MORF oligomer. The goal of this study was to evaluate the influence of streptavidin on the cell transfection properties of the carrier and the antisense properties of the MORF oligomer. **Metbods**: In all cases, the Tc99m radiolabel was placed on the MORF. The model carriers were a tat and a polyarginine peptide and cholesterol. The 25 mer MORF was selected as a suitable test oligomer because of poor cell accumulations in culture when incubated naked (i.e. without streptavidin and/or carrier). Accumulations in the survivin positive MCF-7 cells of the antisense MORF as the nanoparticle were compared to the sense MORF nanoparticle, to the antisense MORF nanoparticle without the carrier and to the naked antisense MORF. **Results**: The MORF/streptavidin/tat and MORF/streptavidin/polyarginine nanoparticles accumulated about 10 fold more than the MORF/streptavidin and the naked MORF controls after 12 h showing that streptavidin did not interfere with carrier function (the MORF/streptavidin/cholesterol accumulations were lower but stil1 significant). Furthermore, accumulations of the antisense MORF/streptavidin/tat nanoparticle compared to the control sense MORF/streptavidin/tat nanoparticle were significant higher at all times other than 3 h, showing that streptavidin also did not encourage vesicle entrapment nor interfere with antisense function. **Conclusions**: The preparation of oligomer carrier constructs was greatly simplified over covalent conjugations by using streptavidin as a linker. Furthermore, our results suggest that the addition of streptavidin did not interfere with the cellular delivery function of the tat, polyarginine or cholesterol carriers nor with the specific antisense mRNA binding function of the oligomer.

Appendix III. Abstract accepted as poster to the Society for Molecular Imaging and Academy of Molecular Imaging Joint Molecular Imaging Conference in Rhode Island, Sept 2007.

The Accumulation of Antisense MORF Is Improved When Linked to Internalizing Antibodies via Streptavidin and with Evidence of Release

Authors: Yi Wang[1], Xinrong Liu[1], Kayoko Nakamura[2], Atsushi Kubo Kubo[2], Donald J Hnatowich[1,2]

Affiliations: 1. Division of Nuclear Medicine, Department of Radiology, University of Massachusetts Medical School, Worcester, MA, USA and 2. Department of Radiology, Keio University School of Medicine, Tokyo, Japan.

Objective: Delivery of antisense oligomers remains problematic. We are showing that streptavidin (SA) can be used as a convenient linker between biotinylated antisense oligomers and biotinylated antitumor antibodies for improved delivery. This study evaluated the antiHer2 Herceptin as the internalizing antibody, tat as the transfecting peptide and MORF as the antisense oligomer.

Methods: The MORF oligomer against the RhoC mRNA was purchased with a lissamine fluorphore on one end and a primary amine on the other end that was biotinylated. The Herceptin and the 10-mer tat peptide were also biotinylated. The MORF was selected as a test oligomer because of poor cell accumulations in culture when incubated without streptavidin and/or carrier. Accumulations of the antisense MORF as the nanoparticle in the SUM190 (Her2+) cells and SUM149 (Her2-) cells were measure by live cell confocal study, and the efficiencies of transmembrance delivery of the MORF measured with Herceptin or tat peptide under identical conditions. The fluorophore label was replaced on the MORF with ^{99m}Tc and HPLC of lysed cells and media used to estimate the chemical form of the radiolabel.

Results: When incubated at concentration as low as 10 nM, the MORF/SA/Herceptin accumulated more effectively in cells than MORF/SA/tat but in both cases after 6 h the MORF distributed uniformly in the cytoplasm without apparent entrapment. HPLC analysis of the cytoplasm at 6 to 8 h showed that a large percentage of labeled MORF was present free and therefore was released from the SA. Free labeled MORF was also found in the cell media.

Conclusions: Internalizing antitumor antibody such as Herceptin can be used as an efficient carrier for improved cellular delivery of antisense oliogmers or other drugs when linked via streptavidin. Furthermore the antisense oligomer is released within the cell and presumably is therefore unhindered in targeting its mRNA.

1338 *Bioconjugate Chem.* **2007,** *18,* 1338−1343

Simplified Preparation via Streptavidin of Antisense Oligomers/Carriers Nanoparticles Showing Improved Cellular Delivery in Culture

Yi Wang,[†] Kayoko Nakamura,[‡] Xinrong Liu,[†] Naoto Kitamura,[‡] Atsushi Kubo,[‡] and Donald J. Hnatowich*[,†,‡]

Division of Nuclear Medicine, Department of Radiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655, and Department of Radiology, Keio University School of Medicine, Tokyo 160-8582, Japan.
Received January 29, 2007; Revised Manuscript Received April 17, 2007

Objective: Carriers are increasingly now viewed as helpful or even essential to improve cellular uptake in connection with antisense tumor targeting and other applications requiring transmembrane delivery of oligomers. Evaluation of many of the large number of available and potentially useful carriers is limited only by the complexities of preparing the oligomer/carriers by covalent conjugation. However, using streptavidin as a linker between biotinylated carriers and biotinylated antisense oligomers would require only simple mixing for preparation. The goal of this study was to evaluate the preparation and cell accumulation in culture of carrier/streptavidin nanoparticle of an antisense phosphorodiamide morpholino (MORF) oligomer. Methods: The model carriers were cholesterol, a 10 mer HIV-tat peptide, and a 10 mer polyarginine, each having been reported elsewhere to improve cellular delivery of oligomers. The model antisense oligomer was the 25 mer MORF targeting the survivin mRNA. The accumulations of the antisense MORF/carrier nanoparticle were compared to the sense MORF/carrier, to the carrier-free nanoparticles, and to the naked antisense MORF in the survivin-expressing MCF-7 cells. The MORFs and peptides were purchased biotinylated, while the cholesterol was biotinylated in-house. In all cases, the ^{99m}Tc radiolabel was placed on the oligomers. Cell studies were performed at low nM concentration as required for antisense imaging applications and at 37 °C primarily in 1% FBS. Results: Each radiolabeled oligomer/streptavidin/carrier nanoparticle was successfully prepared by careful mixing at a 1:1 molar ratio. As evidence of carrier participation, the radiolabeled MORF showed increased accumulation in cells when incubated as the nanoparticle compared to the carrier-free nanoparticle and by as much as a factor of 11. Accumulation of the antisense MORF/streptavidin/tat nanoparticle was significantly higher than the sense MORF/streptavidin/tat nanoparticle as evidence of specific antisense targeting. Conclusions: The preparation of oligomer/carrier nanoparticles was greatly simplified over covalent conjugations by using streptavidin as a linker. Furthermore, our results suggest that the addition of streptavidin did not interfere with the cellular delivery function of the tat, polyarginine, or cholesterol carriers nor with the specific antisense mRNA binding function of the MORF oligomer.

INTRODUCTION

The results of recent investigations from these laboratories and elsewhere suggest that radiolabeled DNAs and other oligomers for in vivo antisense targeting of tumor may require carriers for improved transmembrane delivery. Fortunately, a large number of potential carriers have been identified primarily for use in cell culture but with increasing reports of successful in vivo use as well (*1*). However, many of the potentially useful carriers can only now be attached to oligomers by covalent conjugation usually requiring involved synthesis, a process that can be challenging (*2*). Two recent reports have described the use of streptavidin to alter the pharmacokinetics of the tat peptide (*3*) and an antisense peptide nucleic acid (PNA) (*4*). We considered that streptavidin could be used in this manner as a linker for radiolabeled antisense oligomer/carrier nanoparticles (*5*). A similar approach was recently described to improve RNA cellular transport in connection with RNAi (*6*). A large number of potential carriers are already available commercially with biotin groups attached, and others with primary amines may be readily biotinylated. In addition to a greatly simplified preparation stabilized by the high binding affinity of biotin for streptavidin, the advantages include the possibility of attaching up to four carriers or oligomers in any combination, since streptavidin possesses four biotin binding sites. This property is illustrated in the artist's conception presented as Figure 1. Toxicities, whether of the streptavidin, oligomer, or carrier, are unlikely to be evident given the low dosages that will be administered in connection with radioimaging, our application of interest. The potential disadvantages include the possibility that streptavidin may interfere with the transmembrane delivery properties of certain carriers and may encourage entrapment of the antisense oligomer in vesicles, thereby restricting its mRNA binding function. In addition, the possibility exists that the addition of streptavidin may unfavorably alter the pharmacokinetics of the oligomer. The goals of this investigation were first to evaluate the preparation of the 1:1 streptavidin nanoparticles and second to address the first two of the above concerns, namely, whether the presence of streptavidin in the nanoparticle will interfere with the ability of carriers to improve cellular accumulations and whether the presence of streptavidin interferes with target mRNA binding by an antisense mechanism.

The three model carriers selected for this initial investigation have each been reported to successfully increase cell membrane permeation in culture. The tat and polyarginine peptides are short cationic peptides with recognized effectiveness as carriers. The tat peptide is the 48−57 residues of the tat protein of HIV-1

* Address correspondence to Donald J. Hnatowich, Ph.D., Professor, Radiology, Division of Nuclear Medicine, Department of Radiology, H2-579, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655,. Tel: (508) 856-4256; Fax: (508) 856-4572; E-mail: donald.hnatowich@umassmed.edu.

[†] University of Massachusetts Medical School.

[‡] Keio University School of Medicine.

10.1021/bc070032c CCC: $37.00
Published on Web 07/03/2007

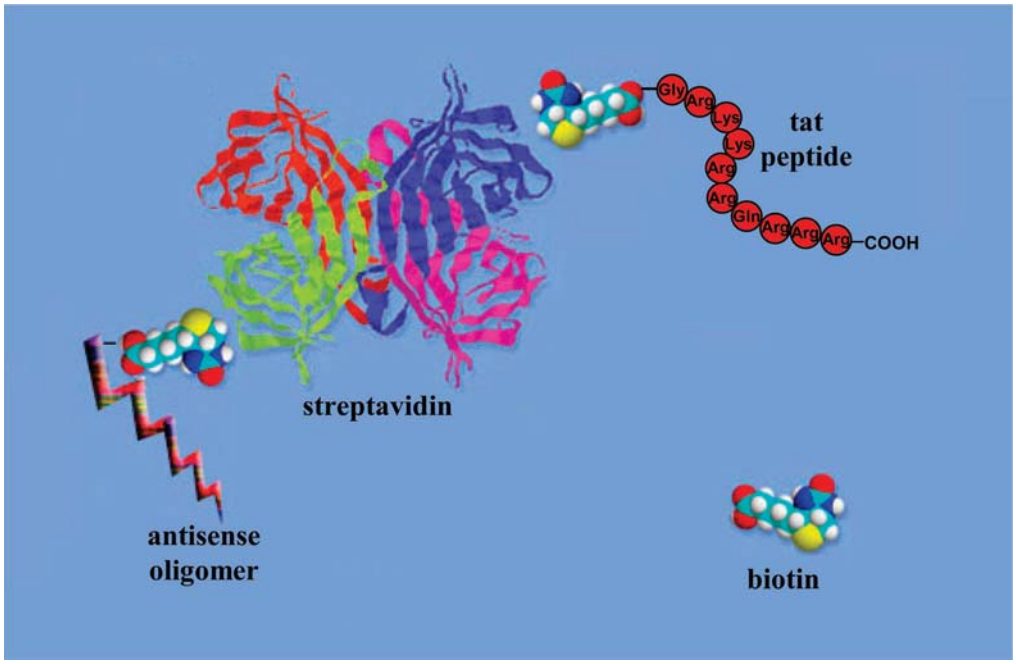

Figure 1. Artist's conception illustrating the composition of the nanoparticle in which the tat peptide and the antisense oligomer have been linked via streptavidin (modified from http://www.arrayit.com/Products/Substrates/SuperStreptavidin/superstreptavidin.html).

with the property of improving accumulations in possibly all cell types by an as yet unknown mechanism (*7*). It has been used as a delivery peptide for the intracellular transduction of large molecules such as proteins, peptides, and MRI contrast agents (*8*), as well as oligomers (*9*, *10*). A polyarginine peptide with similar properties to that of tat was included in this research because of recent reports of effectiveness (*11*−*14*). Finally, the charge neutral cholesterol was selected as a third carrier also because of recent reports of effectiveness (*15*). The model oligomers were both phosphorodiamide morpholinos (MORFs). The study MORF had a base sequence antisense to the 6−30 nucleotides within the survivin mRNA, while the control MORF had the sense sequence.

The influence of the carriers was evaluated by comparing the cell accumulations of the nanoparticle to the carrier-free nanoparticle and to the naked MORF (i.e., without the carrier or the streptavidin).

Determining the antisense properties of the nanoparticle in this investigation requires comment. Since the interest of these laboratories is in antisense imaging, the incubation concentration was fixed at low nanomolarity to avoid target mRNA saturation. While nanomolar concentrations of short double-stranded RNAs can decrease protein expression by RNAi mechanisms (*6*), concentrations in the micromolar range are generally required to decrease protein expression with antisense MORFs and other oligomers (*16*). Accordingly, in this investigation, evidence of antisense targeting was evaluated by comparing cellular accumulations of the antisense to the sense nanoparticles rather than by measurement of survivin protein concentration.

EXPERIMENTAL PROCEDURES

Chemicals and Oligomers. The 25 mer antisense MORF (5′-CCA-ACG-GGT-CCC-GCG-ATT-CAA-ATC-T) and the sense control MORF were obtained with a biotin group on the 3′ equivalent end via a 6-aminohexanoic acid linker and a primary amine on the opposite end (GeneTools, Philomath, OR). The S-acetyl NHS−MAG3 was synthesized in-house (*17*) and its structure confirmed by elemental analysis, proton nuclear magnetic resonance, and mass spectroscopy. The tat (biotin-linker-G-R-K-K-R-R-Q-R-R-R) and polyarginine peptide were both 10 mer and were purchased HPLC purified as the native L-isomer with the biotin attached to the amine end via a 6-aminohexanoic acid linker (21st Century Biochemicals, Marlboro, MA). The biotinylated cholesterol was synthesized by reacting biotinyl-3,6-dioxaoctanediamine (Pierce, Rockford, IL) with cholesteryl choloroformate (Aldrich, Milwaukee, WI). The human breast cancer cell line MCF-7 was a gift from Dr. Dario C. Altieri (Cancer Biology, University of Massachusetts Medical School). This cell line is reported to be a high-level expressor of the survivin mRNA (*18*), and the expression of the survivin mRNA was confirmed by RT-PCR (*19*). Standard chemicals were obtained from various suppliers and used without purification. The ^{99m}Tc-pertechnetate was eluted from a ^{99}Mo−^{99m}Tc generator (Bristol-Myers Squibb Medical Imaging Inc., North Billerica, MA).

Oligomer Conjugation and Radiolabeling. All oligomers were conjugated with S-acetyl NHS-MAG3 via the derivatized amine as previously described (*20*). A solution of 1 mg of oligomer in 200 μL of 0.3 M pH 8.0 HEPES buffer was added to a vial containing 0.7−1.0 mg S-acetyl NHS-MAG3. The vial was vortexed immediately to a clear solution and incubated for 1 h at room temperature. Thereafter, to the solution was added in order 50 μL of 2 M ammonium acetate, 120 μL of freshly prepared 20 mg/mL $SnCl_2 \cdot 2H_2O$/tartrate solution (100 mg/mL sodium tartrate in 0.5 M ammonium bicarbonate, 0.25 M ammonium acetate, and 0.18 M ammonium hydroxide, pH 9.2) with agitation. After heating at 100 °C for 25 min, the mixture was allowed to cool, and absolute ethanol was added to a concentration of 20% (v/v); then, the mixture was purified on a 1 × 20 cm P4 column (Bio-Rad, Hercules, CA) using 0.25 M ammonium acetate as eluant. The peak fractions were pooled and the oligomer concentration quantitated by UV absorbance at 265 nm according to the manufacturer's instructions. The purified samples were stored at refrigerator temperatures. All solutions were passed through a 0.22 μm filter to ensure sterility.

Radiolabeling of each oligomer was achieved by introducing about 0.5 to 1 mCi (20 μL) of ^{99m}Tc-pertechnetate generator eluant into a combined solution consisting of about 15 μL MAG3-conjugated oligomer (10 μg) in 0.25 M NH_4OAc, 45 μL of 0.25 M ammonium acetate, 15 μL of 50 μg/μL Na_2tartrate$\cdot 2H_2O$ in the pH 8.7 buffer, and 5 μL of fresh 4 μg/μL $SnCl_2 \cdot 2H_2O$ in 10 mM HCl containing 1 mg/mL ascorbate

(*20*). The final pH was about 8.5. After vortexing and then heating for 20 min in boiling water, the labeling was confirmed by size exclusion HPLC, or by C18 Sep-Pak (Waters, Milford, MA) in which the first elution with 0.2 M ammonium acetate removes radiolabeled pertechnetate and tartrate, the second elution with 40% acetonitrile removes radiolabeled oligomer while radiolabeled colloids are retained on the Sep-Pak.

Synthesis of Biotinylated Cholesterol. While both the tat and polyarginine peptides were biotinylated by the manufacturer, the biotinylated cholesterol was prepared in-house for this investigation. Briefly, 12 mg of cholesteryl choloroformate (26.7 μmol) in 60 μL anhydrous chloroform was added to a 1.5 mL microfuge tube containing 12 mg (32 μmol) biotinyl-3,6-dioxaoctanediamine in 200 μL anhydrous 1-methyl-2-pyrrolidinone (NMP). The mixture was vortexed to a clear solution. After adding 4 μL diisopropylethylamine (DIEA), the solution was agitated and placed at room temperature for 16 h. The raw product was precipitated as a white powder by addition of 1 mL ethyl ether. The white powder was dissolved in 200 μL NMP and reprecipitated by addition of ethyl ether two more times to remove diisopropylethylamine (DIEA) and unreacted cholesteryl choloroformate. The white powder was dissolved in 400 μL chloroform and washed with 400 μL 0.1 ammonium acetate four times to remove unreacted biotinyl-3,6-dioxaoctanediamine. The chloroform solution was collected, the solvent removed by evaporation over nitrogen, and the product lyophilized at a high vacuum to remove any remained ammonium acetate.

Nanoparticle Preparation and Testing. The streptavidin/tat was prepared by adding very slowly and with vigorous agitation 11.6 μL of tat at 0.5 μg/μL to 20 μL of streptavidin at 10.0 μg/μL both dissolved in 0.5 M NaCl and 0.2 M ammonium acetate solution (molar ratio 1:1). Although the addition was equimolar, the formation of significant concentrations of the 1:2 streptavidin/tat nanoparticle is possible. That the product was free of higher-order tat nanoparticles, i.e., two to four tat peptides per nanoparticle, was confirmed by adding tracer levels of ^{99m}Tc-labeled biotinylated tat peptide to the tat solution before the addition to streptavidin and analyzing the product by size exclusion HPLC with in-line UV and radioactivity detection using 20% acetonitrile in 1 M NaCl, 0.1 M ammonium acetate as eluant at a flow rate 0.6 mL/min. The appearance of a single peak in the UV trace at 280 nm corresponding to a single peak in the radioactivity trace was evidence of complete complexation, since this assay is capable of resolving 1:2 and higher-order nanoparticles (data not presented). Thereafter, the ^{99m}Tc-labeled biotinylated MORF was added to the streptavidin/tat nanoparticle in the identical fashion.

To confirm that the final product was also free of higher-order MORF nanoparticles, i.e., two or three MORFs per streptavidin/tat nanoparticle, the identical preparation was repeated with unlabeled antisense MORF and to the final product was added at tracer levels the complementary DNA (without biotin) radiolabeled with ^{99m}Tc. As before, the appearance by size exclusion HPLC of a single peak in the UV trace at 265 nm was evidence of a successful preparation. As further evidence against higher-order nanoparticles, the nanoparticle was intentionally prepared at a 1:1 tat/streptavidin molar ratio but at a 2:1 MORF/streptavidin molar ratio followed by agarose gel electrophoresis analysis. The complementary phosphodiester DNA was added to each nanoparticle before analysis so that ethidium bromide could be used for detection.

Both the radiolabeled polyarginine and cholesterol nanoparticles were prepared and tested in the identical fashion as was the carrier-free MORF/streptavidin nanoparticle. Radioactivity recoveries were routinely measured in all HPLC analyses and were in all cases 90% or better.

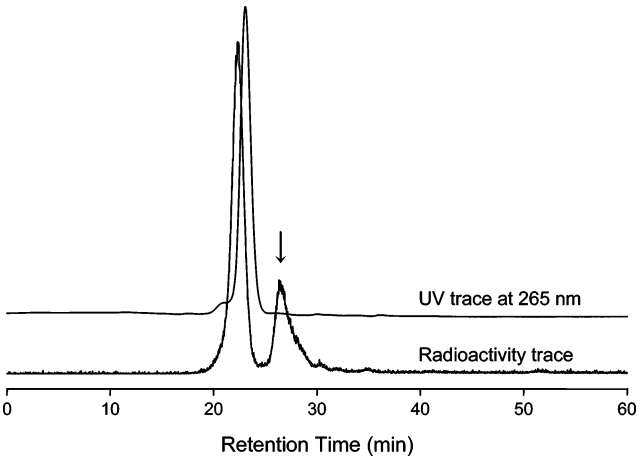

Figure 2. Size exclusion UV trace (265 nm) and radioactivity trace of the antisense MORF/streptavidin/tat nanoparticle radiolabeled by addition of trace ^{99m}Tc-sense DNA (without biotin). The smaller radioactivity peak (arrow) is due to free MORF in the nanoparticle preparation. The small difference in retention time between the UV and radioactivity traces is due to the hybridization of the sense DNA.

Cellular Accumulation Studies. The MCF-7 cells growing in monolayers were expected to be in log phase at the time of the study. Cells were seeded at $(3-4) \times 10^5$ cells per well in 12-well plates. In quadruplet, 1.0 mL of the ^{99m}Tc-labeled oligomer dissolved in 1% FBS DMEM medium was added to each well and the cells incubated at 37 °C in humidified 5% carbon dioxide for different times. The concentration of oligomer was at all times 8 nM to avoid mRNA saturation. The cells were harvested by lysis with 0.2 M NaOH and 1% SDS after removing the medium and washing twice with PBS.

RESULTS

Nanoparticle Preparation and Testing. Figure 2 presents both the UV (265 nm) and radioactivity traces obtained by size exclusion HPLC of the antisense MORF/streptavidin/tat nanoparticle to which trace ^{99m}Tc-labeled sense DNA (without biotin) was added. The radiolabeled sense DNA will hybridize with the antisense MORF in each of its forms and provide a radioactivity peak for each. As shown, only one peak corresponding to the antisense MORF/streptavidin/tat nanoparticles appears in the radioactivity profile and corresponds to the UV trace. Therefore, the result confirms the absence of higher-order MORF/streptavidin/tat nanoparticles. As shown in the figure, a second radioactivity peak is present with about 15% of the radioactivity. This peak is due to free antisense MORF in the preparation resulting from the absence of biotin on a fraction of the MORF provided by the manufacturer. Accordingly, the antisense MORF/streptavidin/tat nanoparticles also showed the same 15% impurity. Since the cellular accumulation of the uncharged naked MORFs is negligible (see below and ref 21), the accumulation of this radiocontaminant will lower the percent accumulations but may otherwise be ignored in the cell accumulations studies. By the same analysis method, biotin was missing on about 20% of the sense MORF.

Figure 3 presents the results of agarose gel electrophoresis of four nanoparticles prepared at 1- and 2-fold molar excess of MORF. The complementary phosphodiester DNA was added to each nanoparticle before analysis so that ethidium bromide could be used for visualization (thus, the nanoparticle with a 2-fold molar excess of MORF will also have twice the DNA therefore twice the charge). The obvious difference in migration of the nanoparticle with two MORFs per streptavidin compared to one (lanes 4 versus 2 without tat and lanes 5 versus 3 with tat) shows that preparation at an intentional 2-fold molar excess

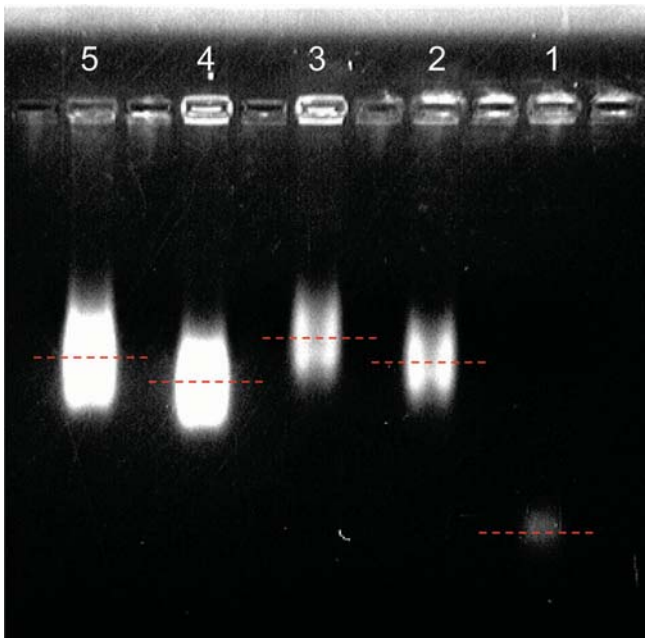

Figure 3. Agarose gel showing the relative positions of naked MORF (lane 1), MORF/streptavidin nanoparticle prepared at a 1:1 molar ratio (lane 2), MORF/streptavidin/tat prepared at a 1:1:1 molar ratio (lane 3), MORF/streptavidin prepared at a 2:1 molar ratio (lane 4), and MORF/streptavidin/tat prepared at a 2:1:1 molar ratio (lane 5). The dotted line in each lane defines the center of each band.

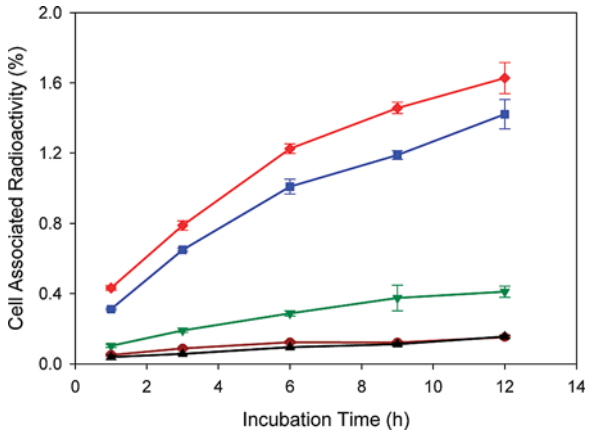

Figure 4. Percent accumulations in MCF-7 cells over time of radiolabeled antisense MORF when incubated as the tat (squares, ■), polyarginine (diamonds, ♦), or cholesterol (inverted triangles, ▼) streptavidin nanoparticles and as the carrier-free nanoparticle (circles, ●) and naked MORF (triangles, ▲) controls. ($N = 4$; error bars indicate one standard deviation).

provides a nanoparticle of higher order compared to nanoparticles prepared under the conditions of this investigation.

MORF Cellular Accumulation Studies. No evidence of cellular toxicity by growth delay was observed in any cell study of this investigation. Results are presented in Figure 4 as the average percent accumulation ($N = 4$) for each of three study MORF nanoparticles and both the carrier-free streptavidin nanoparticle and naked MORF controls in MCF-7 cells at 1, 3, 6, 9, and 12 h of incubation. As shown, adding the carrier increases the accumulations over controls by about 9-fold for tat and 11-fold for polyarginine and somewhat less for cholesterol. The identical accumulations for the MORF/streptavidin nanoparticle compared to the naked MORF suggest that the streptavidin was not responsible for these improved accumulations.

Figure 5 present accumulations in MCF-7 cells over time of the antisense and the sense control MORF/streptavidin/tat

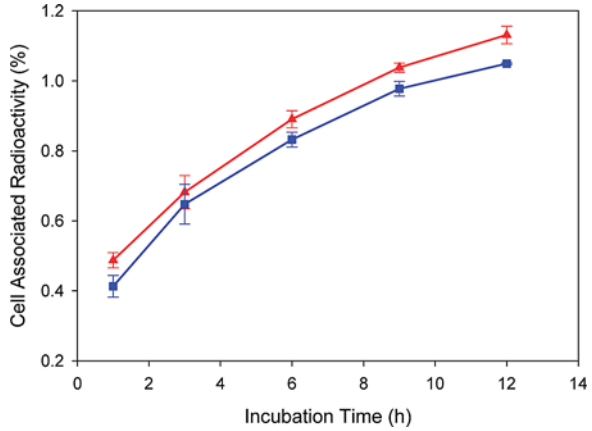

Figure 5. Percent accumulations over time of the antisense MORF/streptavidin/tat nanoparticle (triangles, ▲) compared to that of the control sense MORF/streptavidin/tat nanoparticle (squares, ■). The difference is significant (Student's t test, $p < 0.05$) at all times other than 3 h, providing evidence for specific binding to target mRNA of the nanoparticle and therefore escape from entrapment. ($N = 4$; error bars indicate one standard deviation).

nanoparticles. The differences are significant (Student's t test, $p < 0.05$) at all times other than 3 h, providing evidence for preserved antisense binding function of the nanoparticle and therefore escape from entrapment.

DISCUSSION

In general, all in vivo applications of radiolabeled DNAs and their analogues such as MORFs (collectively: oligomers) are currently characterized to varying degree by the same limitation: poor pharmacokinetic delivery to the target following intravenous administration and, in the case of applications with intracellular targets, poor delivery through cell membranes (*22*). Attempts to increase delivery are now often focused on the use of oligomer/carrier constructs in which the carrier is intended to improve pharmacokinetics, for example, by adding an antitumor antibody to direct the nanoparticle to its target, or more commonly, adding a carrier intended to improve cellular accumulations by chaperoning the oligomer through cellular membranes primarily in vitro but increasingly now in vivo as well.

Charged oligomers such as the DNAs have the advantage that they may be complexed to charged carriers such as cationic peptides or liposomes simply by electrostatic binding (*23*, *24*). While a number of cationic carriers for DNAs are commercially available, the vast majority of these have been designed for in vitro delivery in cell culture and are not recommended by the manufacturers for use in serum environments such as those experienced in vivo. We have achieved largely unfavorable in vivo results using two commercially available cationic carriers with DNAs prepared simply by electrostatic binding (*25*). In cases of uncharged oligomers such as peptide nucleic acids (PNAs) and MORFs, covalent conjugation is necessary at present for carrier attachment, and the synthesis of these nanoparticles can prove difficult (*2*). Even the solid-phase synthesis of PNA/peptide carrier nanoparticles is not without its own difficulties and in our hands has led to products of questionable quality.

In this investigation, the use of streptavidin to prepare the carrier and oligomer nanoparticles performed as anticipated. As shown in Figure 1, the slow addition at a 1:1 molar ratio with continuous agitation, first of the biotinylated carrier to streptavidin, and subsequently of the biotinylated MORF to the streptavidin/carrier, provided a product free of both 1:2 com-

plexes. Since the nanoparticle is expected to dissociate in situ, in all cases, the radiolabel was on the MORF rather than the carrier or streptavidin.

One potential drawback to the use of streptavidin as linker may be a susceptibility to dissociation by endogenous biotin. However, we do not expect endogenous biotin to interfere by displacing biotinylated agents from streptavidin. In preliminary studies, D-biotin at 56 mM was incubated with the streptavidin/tat or streptavidin/MORF used in this investigation at 0.028 mM for 1 h in 37 °C PBS with no evidence of displacement. Since the serum concentration of endogenous biotin in a mouse is about 10 nM (*26*), the displacement from streptavidin by endogenous biotin should be negligable for tat or other biotinylated agents.

In addition to evaluating the ease with which carriers and antisense oligomers may be joined via streptavidin as a convenient linker, a goal of this investigation was to evaluate whether the function of the carrier and the function of the antisense MORF was adversely influenced by the presence of the streptavidin. As shown in Figure 4, the presence of each of the carriers and the streptavidin significantly increased accumulations compared to that of both the naked MORF and the carrier-free nanoparticle and by as much as a factor of 11. Therefore, the streptavidin may not have adversely influenced the function of the carrier to increase cell permeation. As shown in Figure 5, the accumulation of the antisense MORF was statistically higher than the accumulation of the control sense MORF when both were incubated with the MCF-7 cells as the streptavidin/tat nanoparticles. Therefore, the streptavidin may not have adversely influenced the antisense binding properties of the MORF for its mRNA target.

This study is a more extensive evaluation of the use of streptavidin as a linker between biotinylated oligomers and carriers than earlier work from this laboratory (*5*). Recently, Chu et al. described their use of streptavidin as a bridge between a double-stranded siRNA and an RNA aptamer functioning as a cellular delivery agent (*6*). The streptavidin was found by PCR to have little or no effect on the ability of the siRNA to inhibit gene expression. Thus, both by gene expression analysis and by radioactivity accumulations in two difference cells in culture, the evidence is against an adverse influence of streptavidin on function.

CONCLUSION

In conclusion, an advantage to the use of streptavidin as linker is in the simplicity of nanoparticle preparation and therefore the ease with which different nanoparticles may be made and tested in contrast to covalent conjugation. Obviously, the same approach may be used to link tumor targeting agents such as antitumor antibodies as well as antitumor peptides to antisense oligomers with or without carriers. Finally, no evidence was obtained in this investigation that the presence of streptavidin in the nanoparticle interferes with the ability of carriers to improve cell membrane permeation nor with the antisense properties of the oligomers. Taken together, the results of this investigation suggest that further studies in tumored animals are justified to address the issue of whether the streptavidin adversely influences the pharmacokinetic properties of the oligomers.

ACKNOWLEDGMENT

The authors wish to thank Dr. Dario C. Altieri (Cancer Biology, University of Massachusetts Medical School, Massachusetts) for providing the MCF-7 cell line. This research was supported in part by the Office of Science (BER), U.S. Department of Energy, grant no. DE-FG02-03ER63602, the National Institute of Health, grant no. SR21CA100092-02, the Congressionally Directed Medical Research Programs (CDMRP), grant no. W81XWH-06-1-0649, and the 21st Century Center of Excellence (COE) Program entitled "Establishment of individualized cancer therapy based on comprehensive development of minimally invasive and innovative therapeutic methods (Keio University)" from the Japanese Ministry of Education, Science, Sports and Culture. The authors also thank Grant-in-Aid for Scientific Research (19591434, 17659374, 19659315).

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BC070032C